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DOCUMENTATION PAGE

AD-A203 364

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1a

1b RESTRICTIVE MARKINGS
N/A

2a
N/A

3 DISTRIBUTION / AVAILABILITY OF REPORT
Distribution Unlimited.

2b DECLASSIFICATION / DOWNGRADING SCHEDULE
N/A

4 PERFORMING ORGANIZATION REPORT NUMBER(S)
Wayne State University

5 MONITORING ORGANIZATION REPORT NUMBER(S)
N/A

6a NAME OF PERFORMING ORGANIZATION	6b OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION
Wayne State University	N/A	Office of Naval Research

6c ADDRESS (City, State, and ZIP Code)
Department of Biological Sciences
Wayne State University
Detroit, Michigan 48202

7b ADDRESS (City, State, and ZIP Code)
800 N. Quincy Street
Arlington, Virginia 22217-5000

8a NAME OF FUNDING / SPONSORING ORGANIZATION	8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
Office of Naval Research	ONR	N00014-86-K-0634

8c ADDRESS (City, State, and ZIP Code)
800 N. Quincy Street
Arlington, Virginia 22217-5000

10 SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO	PROJECT NO	TASK NO	WORK UNIT ACCESSION NO
61153N	RR04108	rr1F010	

11 TITLE (Include Security Classification)
(U) Influence of Neuroendocrine Mediators on Phagocyte Function

12 PERSONAL AUTHOR(S)
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13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final	FROM 7/1/86 TO 11/30/88	December 1988	16

16 SUPPLEMENTARY NOTATION

17 COSATI CODES		
FIELD	GROUP	SUB-GROUP
08		

18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Stress, Macrophage, Opioid, Adrenergic

19 ABSTRACT (Continue on reverse if necessary and identify by block number) The objective of this research program was to explore the effects of certain neuroendocrine mediators on macrophage function. Treatment with met-enkephalin led to a flattened cell morphology with many linear and punctate microfilaments in the cytoplasm. These physical changes in cell structure were accompanied by dose-dependent increases in phagocytosis, spreading and adherence whereas cytolysis was not affected. In contrast, epinephrine treatment led to a spherical cell morphology and a dense cortical band of F-actin. Macrophage phagocytosis and spreading were decreased in a dose-dependent fashion whereas adherence and cytolysis were unaffected. Propranolol reversed the effects of epinephrine. The effects of epinephrine were mimicked by cAMP analogs and forskolin. Combined experiments using optimal met-enkephalin doses and various doses of epinephrine gave results identical to that of epinephrine alone. These receptor signaling pathways may interact via protein kinases. Protein kinase C activators and inhibitors enhance and depress macrophage spreading, respectively. Cyclic AMP antagonizes the action of protein kinase C activators. Protein kinase systems may transduce multiple neuroendocrine signals into physiological responses via effects on granules and the cytoskeleton.

20 DISTRIBUTION / AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS

21 ABSTRACT SECURITY CLASSIFICATION
(U)

22a NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Dr. J. A. Majde	(202) 696-4055	ONR

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted
All other editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE

DISTRIBUTION STATEMENT A
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FINAL REPORT

Influence of Neuroendocrine Mediators on Phagocyte Function

ONR Contract No. N00014-86-K-0634

A. INTRODUCTION

The macrophage cell surface expresses more than 40 distinct receptor activities. These receptors bind a wide spectrum of physiological ligands, including lipoproteins, hormones, growth factors, neurotransmitters, antibodies, complement components, connective tissue molecules, and lysosomal enzymes. In vitro studies of cellular responses to these ligands typically analyzed the interaction of one ligand with one receptor. However, in the complex physiological environment cells may be simultaneously presented with mutually exclusive signals. For example, histamine receptors promote leukocyte detachment, whereas C3bi receptors promote cell adhesion (1,2). To determine the most important ligand-receptor interactions during specific sets of in vitro conditions, we have studied the responses of phagocytic cells to neuroendocrine ligands that separately evoke opposite physiological effects.

Various neuroendocrine mediators are capable of enhancing or depressing phagocyte effector functions (3-14). In general, opioids have been found to enhance effector functions, whereas adrenergic agonists depress effector functions. Furthermore, these stress-related neuroendocrine mediators are stored and secreted together from the adrenal medulla (15-18). It would therefore seem that combinative studies utilizing both opioid and adrenergic agonists would more closely reflect in vivo neuroregulation of macrophage effector function. We herein report individual and combinative studies of the effects of met-enkephalin and epinephrine on macrophage structure and effector functions. A possible interactive membrane signal transduction pathway, which could link individual and combinative receptor binding to macrophage structure and function, is discussed.

B. RESEARCH RESULTS

1. Results from July 1, 1988 - Nov. 1, 1988

a. Studies with Met-Enkephalin and Epinephrine

In most recent Annual Report dated July 1, 1988, we described the ability of met-enkephalin and epinephrine to regulate macrophage morphology, spreading, adherence, and microfilaments. To further characterize the ability of epinephrine to inhibit macrophage spreading in the presence of 10^{-8} M met-enkephalin, we have performed an additional series of dose-response studies. In Figure 1 the percentage of control cell perimeter is plotted at the ordinate. At the left hand side of Figure

1 two bars illustrating the amount of spreading during control conditions and in the presence of 10^{-8} M met-enkephalin are shown. The right hand side of Figure 1 shows a dose-response curve of macrophage spreading in the presence of 10^{-8} M met-enkephalin and 10^{-11} M to 10^{-3} M epinephrine. Epinephrine has no effect on the met-enkephalin-mediated enhancement of spreading at 10^{-11} M. However, spreading is significantly decreased from 10^{-9} to 10^{-3} M epinephrine. It should be noted that the percentage of control cell perimeter from 10^{-9} to 10^{-5} M epinephrine in the absence (14) and presence of met-enkephalin (Figure 1 and ref. 14) are identical. The correspondence of these dose-response curves suggests that a single mechanism is responsible for the inhibition of spreading in the absence and presence of met-enkephalin.

b. Studies on Receptor Signal Transduction

Recent studies (19-30) have suggested that protein kinases A and C play important roles in regulating immune cell function. Our studies with cAMP analogs (14), which are activators of protein kinase A, suggested a role for protein kinases in the neuroendocrine regulation of macrophages. We therefore tested the role of the protein kinases C and A in the non-specific regulation of macrophage spreading by using a series of protein kinase C activators and inhibitors and a cAMP analog.

Scanning Electron Microscopy

Figure 2 shows representative scanning electron micrographs of RAW264 macrophages treated with buffer alone, 1 μM phenylephrine, 3 μM dioctonylglycerol, and 3 μM dioctonylglycerol plus 30 μM dbcAMP. Macrophages were incubated with HBSS or HBSS and reagents for 30 minutes at 37°C. A typical scanning electron micrograph of RAW264 cells incubated in HBSS for 30 minutes at 37°C is shown in Figure 2a. The addition of protein kinase C activators increased macrophage spreading. The addition of 1 μM phenylephrine or 3 μM dioctonylglycerol (Figure 2, panels B and C, respectively), increased macrophage spreading. This qualitative increase in macrophage spreading is consistent with another recent study using PMA (31). We have recently reported that adenylate cyclase agonists or analogs inhibit macrophage spreading (14). To test the interactive behavior of protein kinase C activators and cAMP analogs, we performed scanning electron microscopy studies of cells simultaneously treated with dioctonylglycerol and dbcAMP. Figure 2, panel D shows a representative scanning electron micrograph of RAW264 macrophages exposed to 3 μM dioctonylglycerol and 30 μM dbcAMP. Macrophages treated in this fashion were indistinguishable from cells treated with only 30 μM dbcAMP.

Macrophage Spreading

The effects of the ionophore A23187 and several protein kinase C inhibitors and activators on macrophage spreading was quantitated by measuring the perimeters of individual cells (14). Table I lists the effects of eight reagents on macrophage spreading. Multiple doses of four reagents were tested. The doses of sphingosine, TFP, H-7, and phenylephrine were taken from the literature. These selected doses were

found to possess maximal inhibitory or enhancing activity on macrophage spreading. Macrophages in HBSS were incubated with the reagents at the concentrations listed in Table I for 30 minutes at 37°C. To account for a small day-to-day variability, matched control experiments were performed during each trial. These controls are listed in the second column of Table I. The third column of Table I lists the effect of each reagent found in column one. The protein kinase C inhibitors chlorpromazine, sphingosine, TFP, and H-7 significantly decreased macrophage spreading. In contrast, the protein kinase C activators dioctonylglycerol, phenylephrine, and PMA increased macrophage spreading. Similarly, 1 μM A23187 in a 5 minute assay increased macrophage spreading.

Kinetics of Macrophage Spreading

Figure 3 shows the time-dependence of macrophage spreading. During control conditions macrophage spreading reaches a maximal value by 30 minutes. The protein kinase C activator phenylephrine at 1 μM maximally stimulates cell spreading at 20 minutes ($P < 0.001$ in comparison to controls at 20 minutes). The protein kinase C inhibitor TFP (10 μM) decreases macrophage spreading. The smallest mean macrophage perimeter is reached within 30 minutes ($P < 0.001$ for comparisons of TFP and control at both 20 and 30 minutes). With the exception of A23187, all of the compounds listed in Table I display kinetic properties similar to those of phenylephrine (activators) or TFP (inhibitors). The effect of 1 μM A23187 on spreading was maximal within roughly 5 minutes. However, at longer times or higher doses it becomes toxic. Therefore, the chlorpromazine, sphingosine, TFP, H-7, dioctonylglycerol, phenylephrine, and PMA studies reported in Tables I and II and Figures 2 and 4 were performed at 30 minutes.

Distributions of Macrophage Perimeters During Spreading

The data presented above quantitatively describe the mean perimeter of macrophages during cell spreading. However, they do not show the cell-to-cell variability of these macrophage populations. Figure 4 gives the distributions of macrophage perimeters during 5 experimental conditions. The abscissa is the cell perimeter while the ordinate is the number of cells. The number of cells in each perimeter grouping for three independent experiments were combined then plotted at the ordinate. In addition to controls, two stimulatory (DG and PE) and two inhibitory (TFP and H-7) drugs are shown.

Combinative Effects of Protein Kinase C Activators and dbcAMP

In a previous study we reported that cAMP analogs and agonists inhibit macrophage spreading in the presence of ligands that are known to promote spreading (14). Therefore, we have tested the ability of the cAMP analog dbcAMP to interfere with the increase in spreading mediated by protein kinase C activators. As shown in Table II, 30 μM dbcAMP abrogates the increase in spreading mediated by the protein kinase C activators dioctonylglycerol, PMA, and phenylephrine and the ionophore A23187. Moreover, dbcAMP significantly decreases spreading in the presence of

dioctonylglycerol, PMA, and A23187. Therefore, dbcAMP can interfere with protein kinase C-mediated enhancement of spreading.

2. Results from Entire Contract Period

a. Phagocytosis and Cytolysis

Macrophages have been shown to possess cell surface receptors for opiates and catecholamines. The abilities of these ligands to affect RAW264 macrophage antibody-dependent effector activity directed against sheep red blood cells were tested. Phagocytosis was measured by the uptake of ^{51}Cr labeled erythrocytes and optical microscopy. Cytolysis was measured by ^{51}Cr-release assays. Met-enkephalin increased specific antibody-dependent phagocytosis in a dose-dependent fashion; the optimal dose was found to be 10^{-8} M. Epinephrine diminished phagocytosis in a dose-dependent manner exhibiting a maximal inhibition at 10^{-4} - 10^{-5} M. This inhibition can be blocked by propranolol. The combined effects of simultaneous treatment with met-enkephalin and epinephrine were measured. At the several doses tested, the combined effects of these two ligands on the amount of phagocytosis were equivalent to or more inhibitory than epinephrine alone. Thioglycolate-elicited murine peritoneal macrophages demonstrated similar responses to epinephrine, met-enkephalin, and their combination. Therefore, in vitro models more closely approximating in vivo neuroregulation of macrophage function demonstrate phagocytic inhibition.

b. Spreading, Adherence, Morphology and Cytoskeleton

Cell surface ligand-receptor interactions play a central role in the regulation and expression of macrophage function. Included among these macrophage membrane receptors are the β-adrenergic and opioid receptors. We have studied the abilities of epinephrine, met-enkephalin, forskolin and cAMP analogs to affect macrophage morphology, spreading, and adherence. Cell spreading was quantitated by measuring the perimeters of adherent cell images recorded by videomicroscopy. Epinephrine induced a dose-dependent decrease in macrophage spreading; at 10^{-5} M epinephrine the mean perimeter was 10.4 ± 0.3 μm in comparison to 15.0 ± 1.0 μm for controls. The inhibition of spreading can be blocked by the antagonist propranolol. On the other hand, met-enkephalin induced a dose-dependent increase in macrophage spreading with a perimeter of 18.5 ± 1.0 μm at 10^{-8} M. Since catecholamines and opioids are simultaneously released from chromaffin cells of the adrenal, we have examined the combinative effects due to treatment with both ligands. When macrophages were exposed to 10^{-5} M epinephrine and 10^{-8} M met-enkephalin, cell morphology and spreading were indistinguishable from that due to 10^{-5} M epinephrine alone. The epinephrine dose-response curve in the presence of 10^{-8} M met-enkephalin was similar to that of epinephrine alone. The β-adrenergic receptor is apparently capable of diminishing or abrogating the opioid receptor signal(s). These combinative and epinephrine-mediated effects may be at least partially accounted for by the action of cAMP. Forskolin and the cAMP analogs dbcAMP and Br-cAMP affected cell morphology and spreading in the same fashion as epinephrine. These differences in morphology and spreading behavior were accompanied by changes in the distribution of F-

actin as judged by phalladicin staining and fluorescence microscopy. We suggest that cAMP and microfilaments play important roles in receptor-mediated neuroregulation of macrophage function.

c. Signal Transduction Pathways: Protein Kinases

Protein kinases C and A likely play important roles in membrane signal transduction. To test the role of protein kinases in macrophage spreading, we have measured cell perimeters in the absence and presence of protein kinase C activators, inhibitors, and cAMP analogs. Scanning electron microscopy indicated that macrophages spread extensively in the presence of protein kinase C activators. In contrast, protein kinase C inhibitors and dbcAMP promote a round cell morphology with many surface folds. Quantitative optical microscopy experiments showed that the maximal effects of these reagents were achieved within 30 minutes. The protein kinase C activators dioctonylglycerol (3 μM), phenylephrine (1 μM), and PMA (1 μg/ml) increased macrophage spreading. Similarly, the calcium ionophore A23187 (1 μM) increased spreading. In contrast, the protein kinase C inhibitors chlorpromazine (30 μM), sphingosine (10 μM), TFP (10 μM), and H-7 (10 μM) significantly reduce macrophage spreading. The analog dbcAMP (30 μM) abrogates the effects of protein kinase C activators. These data suggest that protein kinases participate in the regulation of macrophage spreading. Furthermore, the protein kinase A activator dbcAMP can inhibit the effects of protein kinase C activators.

C. SELECTED DISCUSSION POINTS

A detailed analysis of every research observation made during the course of this contract would be far too lengthy to be included in this report. However, two particularly important points will be presented below.

1. Neuroendocrine Control of Macrophage Functions

Several recent studies have considered the role(s) of receptor binding and cAMP production in controlling cell morphology (32,33). Shain et al. (33) have reported that β-adrenergic receptors of astroglial cells regulate changes in cell morphology. Isoproterenol causes astroglial cells to change from an epithelial-like to a stellate morphology. Using a different cell system, we have shown that β-adrenergic ligands influence membrane ruffling and cytoplasmic spreading.

It is well-known that the β-adrenergic receptor is linked to cAMP generation in many cell types, including macrophages (34,35). We therefore tested the ability of exogenous cAMP analogs to affect macrophage morphology and spreading behavior. The analogs dbcAMP and Br-cAMP induced membrane ruffling, cell rounding, and diminished spreading. Furthermore, foreskolin stimulated similar changes in RAW264 macrophage activity. This suggests that cAMP may be a participatory factor in transducing ligand-receptor interactions into altered cell appearance and behavior.

The ability of cAMP to affect cell morphology and spreading is likely

mediated by cytoskeletal interactions. We have shown dramatic differences in the distribution of F-actin within macrophages treated with epinephrine, met-enkephalin, and cAMP analogs (14). The NBD-phallacidin was distributed throughout cells two fashions: (1) as punctate and linear fluorescence and (2) as a circular cortical layer about a cell's perimeter. We have shown that specific ligands such as epinephrine can affect the distribution of actin within macrophages. Furthermore, the ability of cAMP analogs to mimic the action of epinephrine suggests that cAMP could provide a link between receptor and cytoskeleton. Hamachi et al. (36) have shown that dbcAMP: (1) promotes F-actin disassembly and (2) inhibits actin polymerization in macrophages treated with fmet-leu-phe. Since polymerization of G-actin to F-actin is required for macrophage spreading and phagocytosis (37,38), an interaction between cAMP and the cytoskeleton may account for our observations of macrophage morphology, spreading, and our previous study of phagocytosis (13).

Although the above discussion has focused on the potential role of cAMP, additional factors participate in opioid receptor function. Calcium is thought to participate in intracellular signaling of the opioid receptor in this system (4). Kruskal et al. (39) have reported that intracellular calcium levels rise prior to leukocyte spreading. Furthermore, the calcium ionophore A23187 stimulates macrophage spreading (31). Since calmodulin is associated with macrophage microfilaments (40), this may provide one regulatory linkage.

The combinative experiments described above show that epinephrine is capable of inhibiting the functional capacity of the macrophage opioid receptor. Our data suggest that cAMP participates in this process. It seems likely that protein kinase A also plays an important role in this event (30). The macrophage regulatory pathway may be similar to that of platelets (30); cAMP agonists or analogs may antagonize the promotion of cell functions involving the turnover of inositol phospholipids and protein kinase C. This suggestion is consistent with our observations reported above.

A proposed molecular pathway accounting for these individual and combinative effects is shown in Figure 5. Epinephrine binds to the β-adrenergic receptor thereby yielding intracellular cAMP. The cAMP activates protein kinase A. Protein kinase A is proposed to inhibit opioid signal transduction, actin polymerization, and granule release. In the absence of β-adrenergic ligands, met-enkephalin or other ligands can bind to the opioid receptor thereby upregulating microfilaments and cell function.

2. Physiological Setting

In addition to catecholamines, chromaffin cells of the adrenal synthesize and store met-enkephalin and leu-enkephalin (18). These mediators are simultaneously released from chromaffin cells (15-18,41). Therefore, the simultaneous treatment of macrophages with catecholamines and enkephalins may more closely reflect physiological conditions, particularly those of stress. As we have previously emphasized (13), a

cell's response to multiple receptor ligation is not a simple superposition of their individual effects. Based upon their sensory input, cells must transduce conflicting signals into a coherent physiological response. In Figure 6 we show a potential model of the physiological setting of these ligand-receptor interactions. Engagement of only the opioid receptor leads to enhanced macrophage function, as described above and by Petty and Berg (13). This also leads to inhibition of chromaffin cell granule release (42) and its consequent down-regulation of macrophage function (see also ref. 13). On the other hand, stimulation of chromaffin cell nicotinic receptors leads to release of catecholamines and enkephalins; this condition, at least in vitro, leads to down-regulation of macrophage function. The combinative studies described above suggest that ligation of the β-adrenergic receptor plays a central role in regulating macrophage behavior.

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Table I. Effects of Protein Kinase C Activators and Inhibitors on Macrophage Spreading

drugs/compounds		cell perimeter (μm) controls	addition	n[a]	P
chlorpromazine	(300 μM)	15.7 ± 0.5	10.3 ± 0.1	3	=.001
	(30 μM)		13.8 ± 0.7	3	=.02
	(3 μM)		16.0 ± 0.4	3	ns[b]
sphingosine	(10 μM)	15.1 ± 0.3	11.1 ± 0.4	5	<0.001
TFP	(10 μM)	15.5 ± 0.2	10.6 ± 0.3	3	<0.001
H-7	(10 μM)	15.5 ± 0.3	11.7 ± 0.3	3	<0.001
dioctonylglycerol	(30 μM)	14.8 ± 0.2	18.0 ± 1.0	3	=0.001
	(3 μM)		19.1 ± 1.1	3	<0.001
phenylephrine	(1 μM)	15.1 ± 0.3	19.9 ± 0.4	4	<0.001
PMA	(1 μg/ml)	16.0 ± 0.7	20.9 ± 1.1	4	<0.001
	(0.1 μg/ml)		15.4 ± 0.4	3	ns
	(10 ng/ml)		15.6 ± 0.2	3	ns
A23187[c]	(1 μM)	15.6 ± 0.4	19.3 ± 0.5	3	<0.001
	(0.1 μM)		15.8 ± 0.6	3	ns
	(10 nM)		17.1 ± 2.1	4	ns

[a]number of independent trials

[b]ns= not significant

[c]These assays were conducted at 5 min.

Table II. Effect of dbcAMP on Cell Spreading Promoted by Protein Kinase Activators.

	cell perimeter (μm)			
treatment	controls	addition	n	P
dioctonylglycerol (30 μM) + 30 μM dbcAMP	15.1 ± 0.4	13.3 ± 0.09	3	=0.004
PMA (1 μg/ml) + 30 μM dbcAMP	14.7 ± 0.5	12.4 ± 0.7	4	=0.004
A23187 (1 μM) + 30 μM dbcAMP	14.7 ± 0.5	12.2 ± 1.0	4	=0.006
phenylephrine (10 μM) + 30 μM dbcAMP	15.1 ± 0.4	16.6 ± 0.4	3	ns

Figure Legends

Figure 1. The effects of epinephrine on macrophage spreading in the presence of 10^{-8} M met-enkephalin are shown. The mean cell perimeter is given as a percentage of control at the ordinate. The left hand side shows two bars illustrating the spreading of macrophages during control conditions (bark bar) and in the presence of 10^{-8} M met-enkephalin (hatched bar). The right hand side shows the dose-dependent inhibition of spreading in the presence of epinephrine at 10^{-11} to 10^{-3} M.

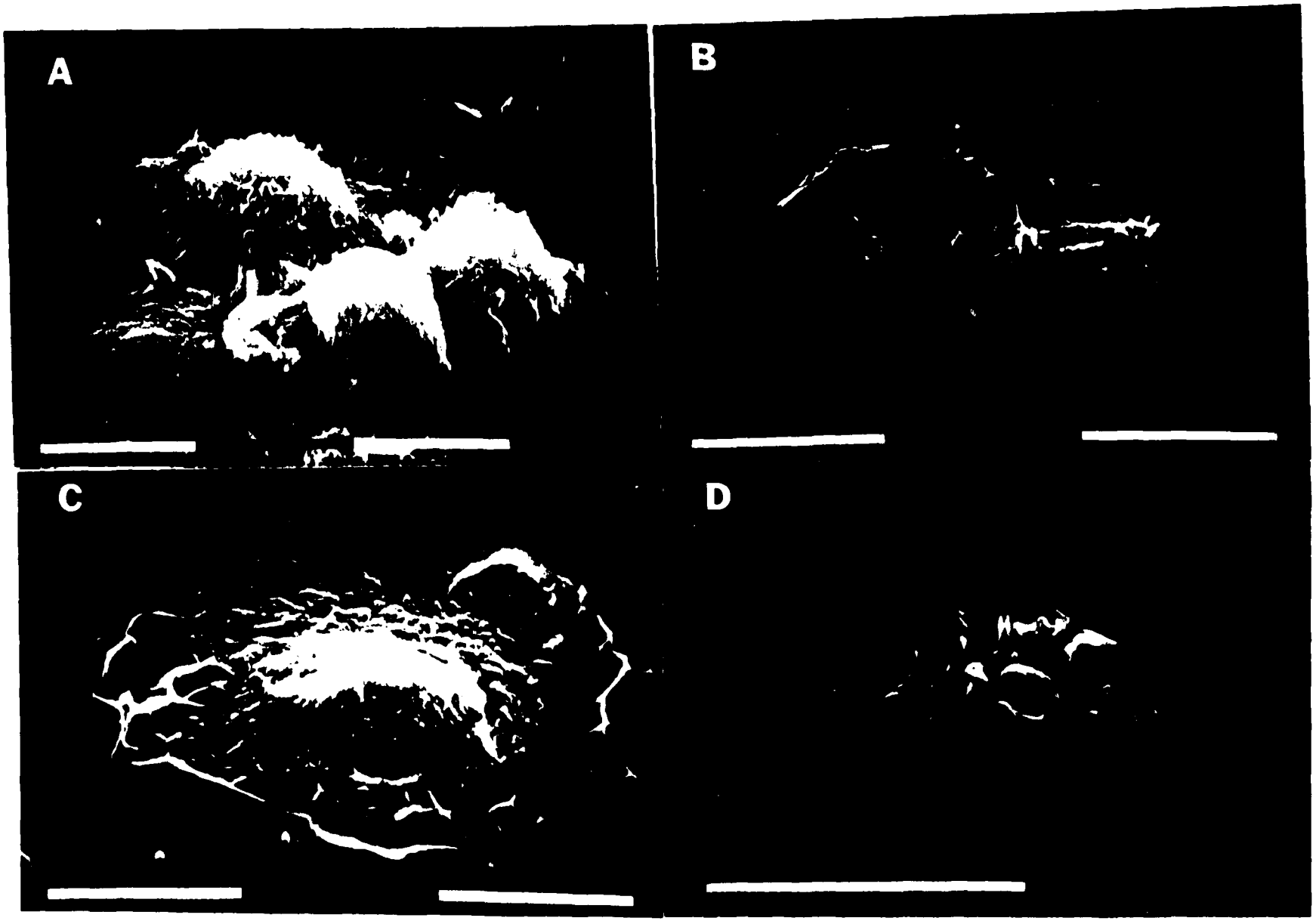
Figure 2. Representative scanning electron micrographs of RAW264 macrophages exposed to buffer alone or to buffer and various compounds are shown. Cells were treated with buffer or buffer plus reagents for 30 min. at 37°C then prepared for electron microscopy as described in Materials and Methods. Scanning electron micrographs of cells treated with (a) HBSS alone (x 2,980), (b) 1 μM phenylephrine (3,700), (c) 3 μM dioctonylglycerol (3,700), and (d) 3 μM dioctonylglycerol plus 30 μM dbcAMP (5,950) are shown. (Bars = 10 μm).

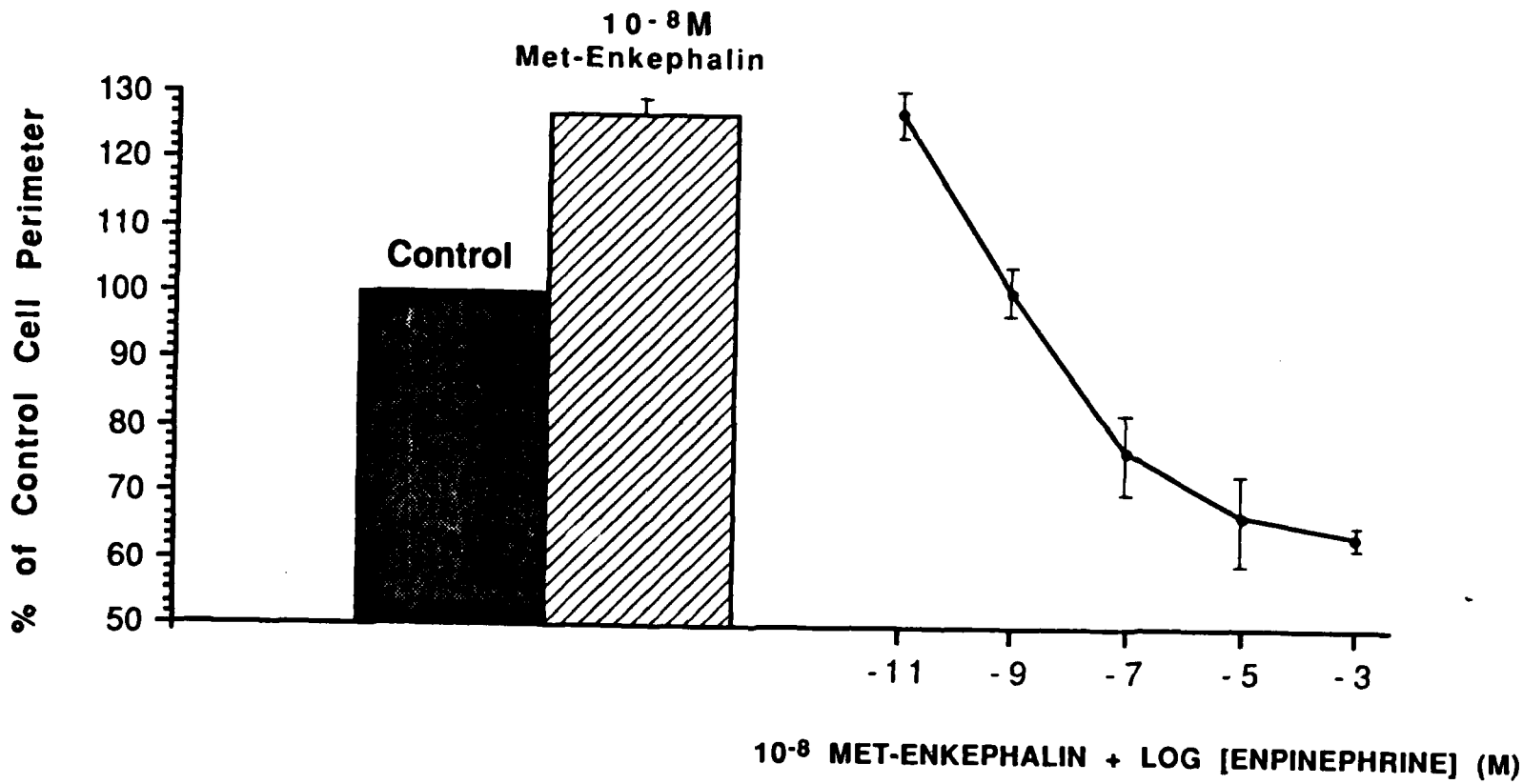
Figure 3. Kinetic studies of macrophage spreading at 37°C are shown. The mean (± s.e.) cell perimeter (μm) is given at the ordinate. The time in minutes is listed at the abscissa. Cells were treated with buffer alone (——), 1 μM phenylephrine (···), or 1 μM TFP. In all cases the maximal effect of the buffer or drug on spreading is reached within 30 minutes.

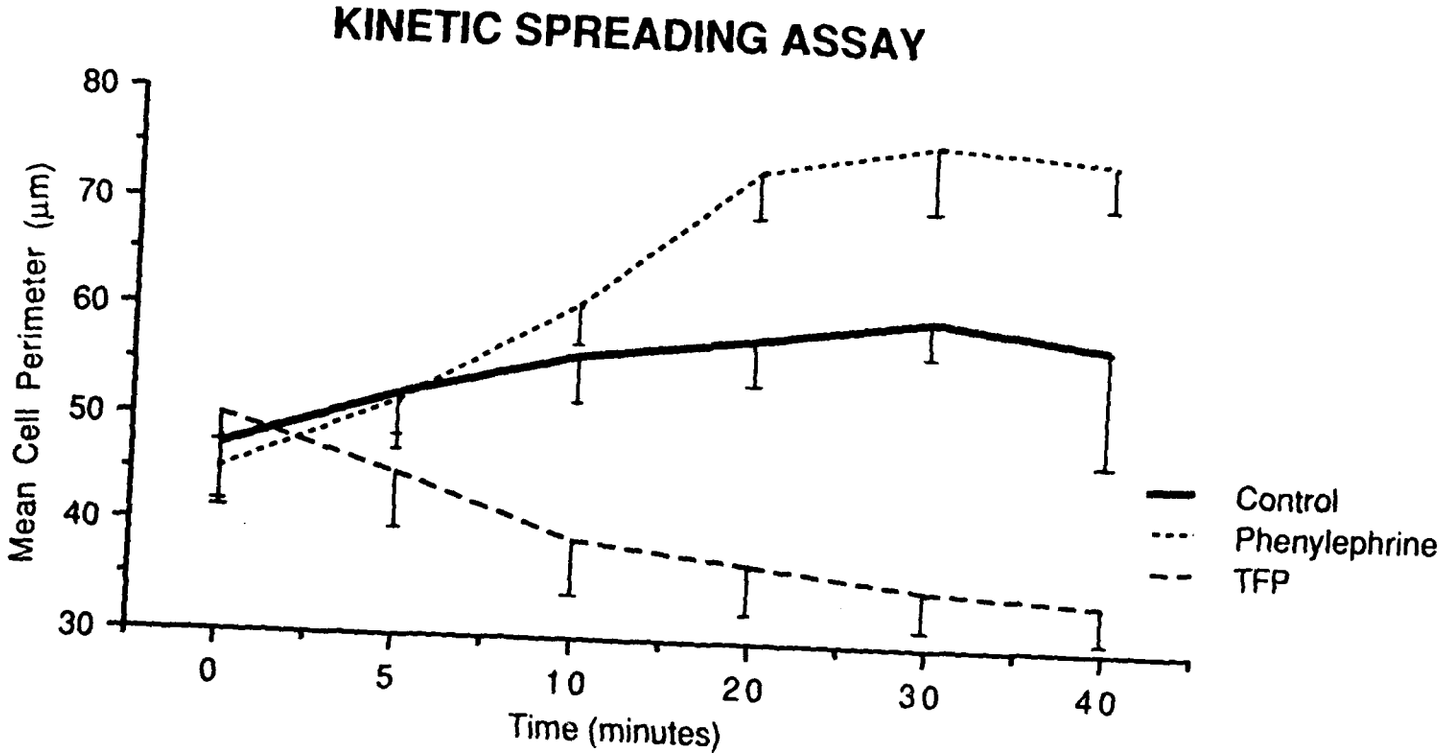
Figure 4. The distribution of macrophage perimeters in the presence and absence of compounds that affect protein kinase C are shown. The cell perimeters were grouped into 17 categories depending upon size. The number of cells in each of these groupings is plotted at the ordinate. Cells exposed to control (——), 10 μM TFP (···), 10 μM H-7 (---), 3 μM DG (---), and 1 μM phenylephrine (–·–) for 30 minutes at 30°C are shown.

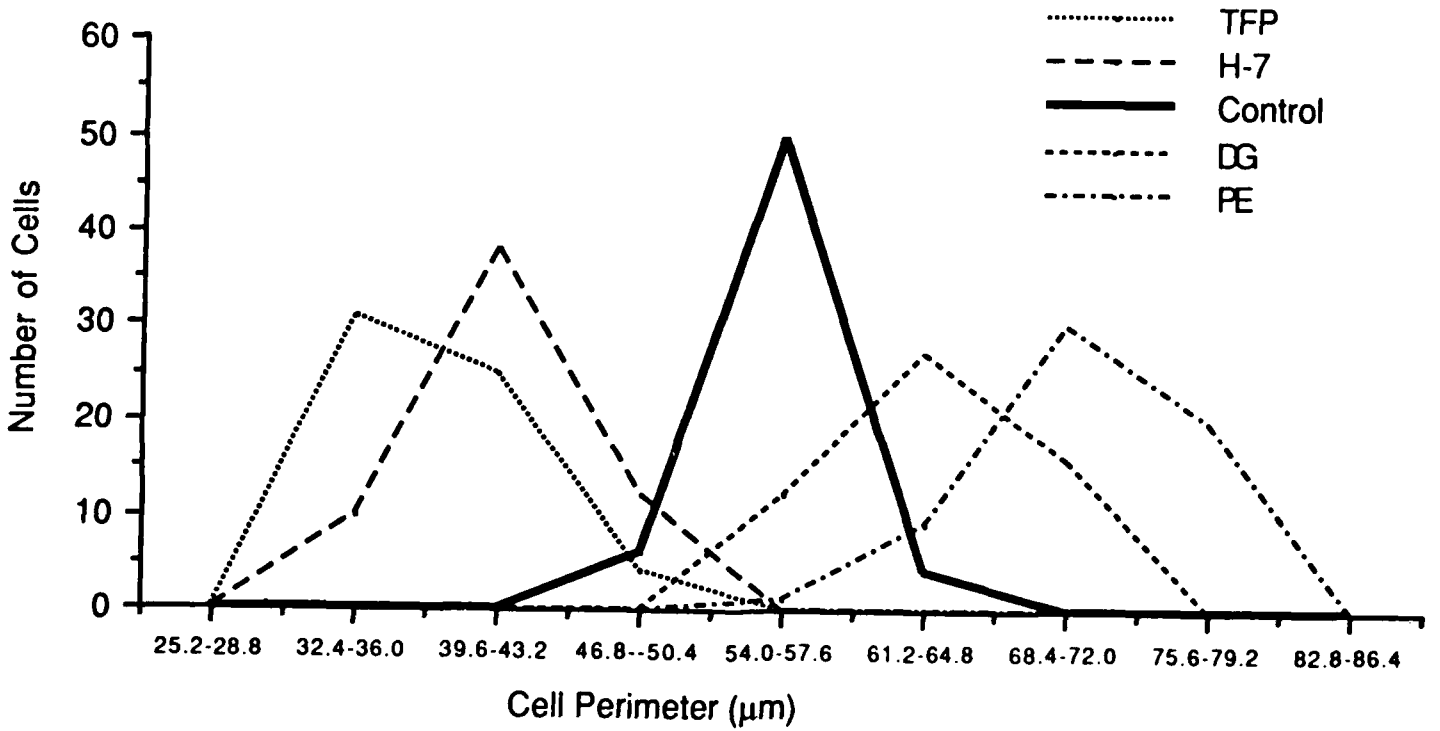
Figure 5. A potential model of the mechanistic pathway of neuroregulation is shown.

Figure 6. A model showing possible in vivo pathways of macrophage neuroregulation is shown. Both up-regulatory (———) and down-regulatory (-----) pathways are illustrated.

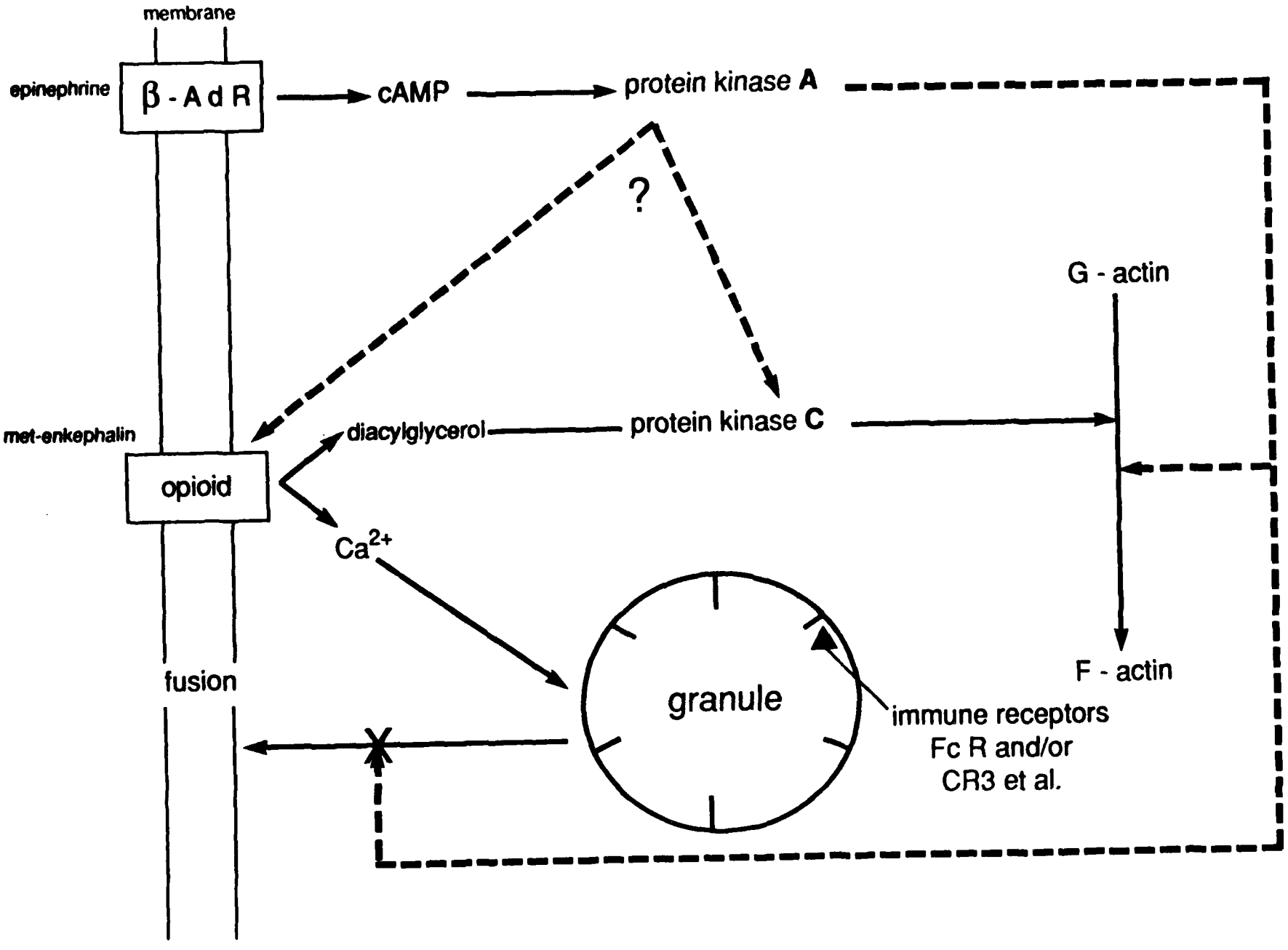

A
B
C
D

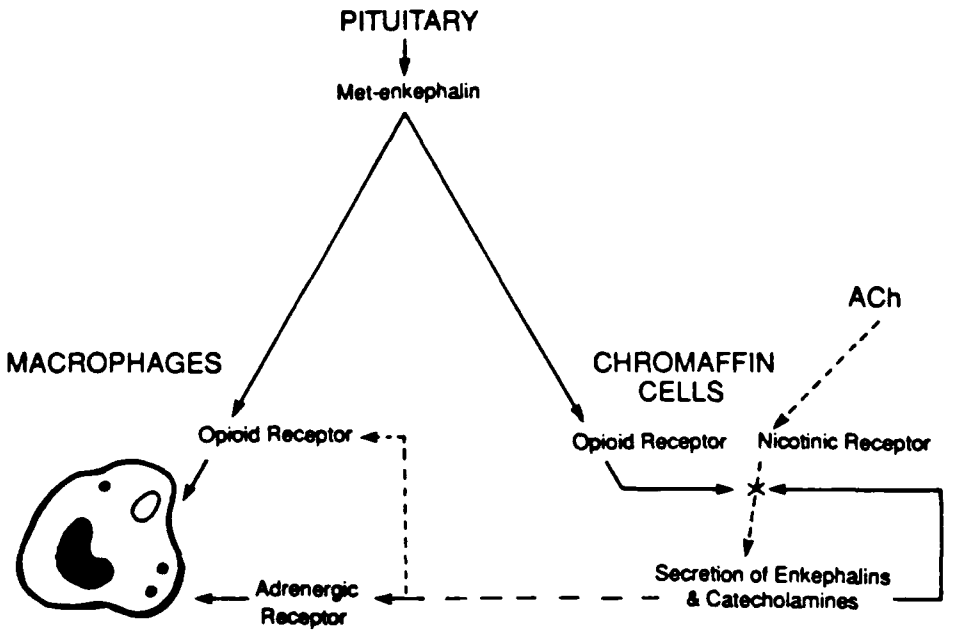

10-8M
Met-Enkephalin
% of Control Cell Perimeter
130
120
110
100
90
80
70
60
50
Control
-11
-9
-7
-5
-3
10-8 MET-ENKEPHALIN + LOG [ENPINEPHRINE] (M)


KINETIC SPREADING ASSAY
Mean Cell Perimeter (µm)
80
70
60
50
40
30
0
5
10
20
30
40
Time (minutes)
Control
Phenylephrine
TFP


TFP
H-7
Control
DG
PE
Number of Cells
60
50
40
30
20
10
0
25.2-28.8
32.4-36.0
39.6-43.2
46.8--50.4
54.0-57.6
61.2-64.8
68.4-72.0
75.6-79.2
82.8-86.4
Cell Perimeter (μm)

Hypothetical Functional Interactions




PITUITARY
Met-enkephalin
ACh
MACROPHAGES
CHROMAFFIN CELLS
Opioid Receptor
Opioid Receptor
Nicotinic Receptor
Adrenergic Receptor
Secretion of Enkephalins & Catecholamines
Up - Regulation
Down - Regulation